

HIV-1 infection and the lack of viral control are associated with greater expression of interleukin-21 receptor on CD8⁺ T cells

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Objectives: Interleukin-21 (IL-21) has been linked with the generation of virus-specific memory CD8⁺ T cells following acute infection with HIV-1 and reduced exhaustion of CD8⁺ T cells. IL-21 has also been implicated in the promotion of CD8⁺ T-cell effector functions during viral infection. Little is known about the expression of interleukin-21 receptor (IL-21R) during HIV-1 infection or its role in HIV-1-specific CD8⁺ T-cell maintenance and subsequent viral control.

Methods: We compared levels of IL-21R expression on total and memory subsets of CD8⁺ T cells from HIV-1-negative and HIV-1-positive donors. We also measured IL-21R on antigen-specific CD8⁺ T cells in volunteers who were positive for HIV-1 and had cytomegalovirus-responding T cells. Finally, we quantified plasma IL-21 in treatment-naïve HIV-1-positive individuals and compared this with IL-21R expression.

Results: IL-21R expression was significantly higher on CD8⁺ T cells ($P = 0.0256$), and on central memory ($P = 0.0055$) and effector memory ($P = 0.0487$) CD8⁺ T-cell subsets from HIV-1-positive individuals relative to HIV-1-negative individuals. For those infected with HIV-1, the levels of IL-21R expression on HIV-1-specific CD8⁺ T cells correlated significantly with visit viral load ($r = 0.6667$, $P = 0.0152$, $n = 13$) and inversely correlated with plasma IL-21 ($r = -0.6273$, $P = 0.0440$, $n = 11$). Lastly, CD8⁺ T cells from individuals with lower set point viral load who demonstrated better viral control had the lowest levels of IL-21R expression and highest levels of plasma IL-21.

Conclusion: Our data demonstrates significant associations between IL-21R expression on peripheral CD8⁺ T cells and viral load, as well as disease trajectory. This suggests that the IL-21 receptor could be a novel marker of CD8⁺ T-cell dysfunction during HIV-1 infection.

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Introduction

CD8⁺ T cells are critical components of the antiviral cellular immune response and play a key role in the resolution of

HIV-1 infection during the acute phase [1–3], where the expansion of HIV-1-specific CD8⁺ T cells has been linked to slower disease progression and control of viremia [2,3]. However, prolonged exposure to antigens derived from

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replicating virus has been shown to lead to immune exhaustion and the emergence of dysfunctional CD8⁺ T cells with reduced cytokine production [4], deterioration of effector function [5] and reduced rates of proliferation [6]. HIV-1 viruses with high replicative capacity have also been linked with increased PD-1 expression and aberrant immune activation of CD8⁺ T cells [7].

Nonetheless, targeting T-cell-mediated immunity may represent the best opportunity for HIV-1 prevention and cure, demonstrated by the exceptional in-vivo control of viral load in the absence of treatment by some individuals [8]. There is a wealth of evidence attributing this durable control to effective HIV-specific CD8⁺ T cells [8–12]. Along with efforts at HIV-1 vaccine development for prevention, adoptive immunotherapy (AIT) through the transfer of ex-vivo expanded HIV-specific T cells has also been exploited in HIV-1 cure strategies [13,14]. Ultimately, both the development of a T-cell-based vaccine or cure strategy for HIV-1 will likely require the restoration and maintenance of T cells for durable immunity.

Cytokines, such as interleukin-7 (IL-7) and interleukin-21 (IL-21), both members of the γ -chain family of cytokines, play an important role in development of the immune response [15–17]. IL-7 is associated with the maintenance of mature T-cell homeostasis and increased T-cell survival [18,19] whilst IL-21 has been linked to the generation of virus-specific memory CD8⁺ T cells [20], promotion of effector function [21,22] and limiting CD8⁺ T-cell exhaustion in viral infections [23]. IL-21-secreting HIV-1-specific CD8⁺ T cells also appear to be preferentially enriched in elite controllers [24].

HIV-1 infection has been linked with decreased expression levels of Interleukin-7 receptor (IL-7R/CD127) on CD8⁺ T cells [25–27]. IL-7R^{low} CD8⁺ T cells exhibit enhanced apoptosis, inferior ex-vivo proliferative capacity and reduced IL-2 production, relative to IL-7R^{high} expressing CD8⁺ T cells from healthy donors [28]. But little is known about IL-21 receptor (IL-21R/CD360) expression on CD8⁺ T cells during HIV-1 infection in humans.

Mouse studies have shown that the absence of IL-21 or its receptor (IL-21^{-/-} and IL-21 receptor^{-/-}) is associated with the emergence of virus-specific CD8⁺ T cells with decreased levels of cytokine production, reduced poly-functionality and increased exhaustion [29–31]. CD8⁺ T cells lacking IL-21R are rapidly lost and this is associated with sustained high viral load during chronic viral infections [32]. These knock-out mouse studies suggest that IL-21 and its receptor are critical to mounting effective CD8⁺ T-cell responses against viral infections.

Whilst the levels of IL-7R expression on CD8⁺ T cells have been associated with control of HIV-1 infection, it is not clear if there is a similar association between IL-21R

expression and HIV-1 disease progression. Here we aim to assess the difference in expression of IL-21R and IL-7R on CD8⁺ T cells from HIV-1-positive and HIV-1-negative volunteers as well as changes in expression associated with HIV-1 viral control.

Methods

Ethics

Work was approved by the local ethics review board. Details have been published elsewhere [33] and on the IAVI open access data warehouse (<https://dataspace.iavi.org/>). Informed consent was obtained from all volunteers prior to the collection of study-related resource.

Description of samples

PBMCs from 20 HIV-1-positive volunteers and eight HIV-1-negative healthy controls were used for this study. Plasma viral load measurements were available for 13 of the 20 HIV-1-positive volunteers (Supplementary Table 1, <http://links.lww.com/QAD/C60>). PBMCs and matching plasma samples were available for 11 of the HIV-1-positive volunteers, which were recruited as part of IAVI Protocol C, a longitudinal natural infection cohort in which participants were followed for up to 7-years post-early infection [33]. PBMCs for the other nine HIV-1-positive and eight HIV-1-negative individuals were prepared by density gradient isolation (Lymphoprep, STEMCELL Technologies Inc, Vancouver, Canada) and provided by the blood bank services in Johannesburg, South Africa.

Descriptive immunology and epidemiology information (including visit dates, visit viral load, CD4⁺ T-cell count and the approximate time the blood sample was collected post-estimated date of infection) for all Protocol C volunteers used in this study are currently available through the IAVI open access data warehouse (<https://dataspace.iavi.org/>). The 11 Protocol C donors have been classified according to their ability to control in-vivo viral load as part of a separate investigation [34]. Briefly, volunteers were identified as low viral load volunteers (LVLVs) or controllers, intermediate viral load volunteers (IVLVs) and high viral load volunteers (HVLVs) or chronic progressors according to the set point plasma viral load calculations for the Protocol C dataset.

Experimental data presented in this manuscript was collected from November 2019 to December 2020.

Measurement of IL-21R and IL-7R expressions by flow cytometry

Frozen PBMC samples were thawed and rested overnight in R20 media [RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum (FCS), penicillin–streptomycin, HEPES, sodium pyruvate and L-glutamine) at 37 °C with 5% CO₂. Samples were washed with PBS and counted

with an automated Vi-Cell cell counter (Beckman, Indianapolis, United States). A total of 3×10^5 cells were added to 96 round bottom plates for each stimulation condition and controls. For each donor, cells were stimulated with MOCK (no peptide, 0.45% DMSO), 0.2 $\mu\text{g/ml}$ Cytomegalovirus peptide pool (CMVpp65), 0.2 $\mu\text{g/ml}$ HIV-1 total protein pool consisting of Gag, Pol, Nev and Env peptide pools belonging to the global potential T-cell epitope (PTE) set (provided by the NIH AIDS reagent programme) or 0.2 $\mu\text{g/ml}$ phytohemagglutinin (PHA, Sigma-Aldrich, Darmstadt, Germany) as positive control. Plates were incubated at 37 °C with 5% CO₂ in the presence of cellular transport inhibitors Monensin (Biolegend, San Diego, United States) and Brefeldin A (Sigma-Aldrich, Darmstadt, Germany) for 6 h. Cells were then washed with PBS before staining with a Live/Dead aqua-amine dye (ThermoFisher Scientific, Stockholm, Sweden) for 20 min in the dark at room temperature, followed by a PBS wash containing 2% FCS (P2) and centrifugation for 4 min at 700g and 4 °C. Human TruStain FcX Blocking Buffer (BioLegend, San Diego, California, USA) was added to all cells and incubated at 4 °C in the dark for 10 min. Fluorescence minus one (FMO) was also set up for stains against CCR7, CD45RO, IL-7R and IL-21R (Supplementary Table 2, <http://links.lww.com/QAD/C60>). Cells were stained at 4 °C in the dark for 20 min followed by two P2 washes. Cells were then fixed and permeabilized with Cytotfix/Cytoperm (BD Biosciences, Oxford, UK) for 20 min at 4 °C in the dark. After two washes with Perm/Wash (BD Biosciences) cells were stained for Tumour Necrosis Factor Alpha (TNF- α) and interferon gamma (IFN- γ) for 20 min at 4 °C, in the dark. Cells were washed twice and resuspended in Perm/Wash before acquisition using a Symphony (BD Biosciences, Reading, UK) multicolour flow cytometer. Data was analyzed using FlowJo v10.6 software (Becton, Dickinson & Company, Portland, Oregon, USA).

Flow cytometry gating strategy

Samples were first gated on lymphocytes followed by single cells (Supplementary Figure 1, <http://links.lww.com/QAD/C60>). The live CD3⁺ population from singlets gate was used to identify CD8⁺ T cells and CD4⁺ T cells. The CD8⁺ gate was used for IFN- γ and TNF- α four quadrant gating on MOCK samples. MOCK gate was then applied to all other IFN- γ and TNF- α gates for that donor. Boolean gating was used to determine total antigen-specific T cells by combining IFN- γ and TNF- α single-positive and double-positive gates. CCR7 and CD45RO FMO gates were used to determine gating on CD8⁺ or CD4⁺-naive and memory populations. IL-7R and IL-21R were gated on CD8⁺ and CD4⁺ populations, total antigen-specific cells as well as all four quadrants of the CCR7 and CD45RO gates on CD4⁺ and CD8⁺ populations. All data was exported as FCS files and analysed in FlowJo v10.6 software (Becton, Dickinson & Company).

Measurement of plasma IL-21 by ELISA

Plasma samples for the 11 Protocol C volunteers were thawed and tested in an IL-21 commercial ELISA kit. The Human IL-21 DuoSet ELISA kit from R&D Systems (Minneapolis, Minnesota, USA) was used to quantify circulating human IL-21 plasma concentrations using the manufacturers protocol. A standard curve was used to interpolate the concentrations of the plasma IL-21 with GraphPad Prism v8 software (GraphPad Software, San Diego, California, USA).

Statistical analysis

All statistical analysis was performed with GraphPad Prism v8 software (GraphPad Software). We assumed that immunological data does not follow a normal distribution, and therefore, applied nonparametric statistical methods throughout this study [35]. To compare differences between two independent groups, we used the Mann-Whitney *U* test. To determine covariance between two groups, we used Spearman's correlation coefficient test. Statistical significance was defined as *P* values less than 0.05. Wherever relevant, a star system was used to denote degrees of statistical significance (**P* < 0.05, ***P* < 0.001, ****P* < 0.0001).

Results

IL-7R expression on CD4⁺ and CD8⁺ T cells in HIV-1-negative and HIV-1-positive PBMCs

Flow cytometry analyses of volunteers showed expected frequencies of T cells, with HIV-1-negative donors having significantly greater frequency of CD4⁺ T cells than HIV-1-positive volunteers (*P* = 0.0014) whereas HIV-1-positive volunteers had significantly greater proportions of CD8⁺ T cells (*P* = 0.0068) (Fig. 1a and b). As has been previously reported [36], there is a down regulation of IL-7R on CD8⁺ T cells in HIV-1-positive volunteers compared with HIV-1-negative individuals (*P* = 0.0163) (Fig. 2b) whereas there was no significant difference in expression on CD4⁺ T cells (Fig. 2a). To determine if this difference in IL-7R expression was limited to specific CD8⁺ T-cell sub-populations, we stratified the CD3⁺CD8⁺ or CD3⁺CD4⁺ cells into naive, central memory, effector memory and terminal compartments based on the expression of CD45RO and CCR7. Each CD8⁺ or CD4⁺ T-cell compartment was then assessed for the expression of IL-7R (Fig. 2c and d). There was no significant difference in expression levels between HIV-1-positive and HIV-1-negative donors in any CD8⁺ or CD4⁺ T-cell subpopulation.

IL-21R expression on CD4⁺ and CD8⁺ T cells on HIV-1-negative and HIV-1-positive PBMCs

In contrast to existing data regarding IL-7R expression on lymphocytes and its associations with HIV-1 infection, it is not known if there is a difference between the levels of

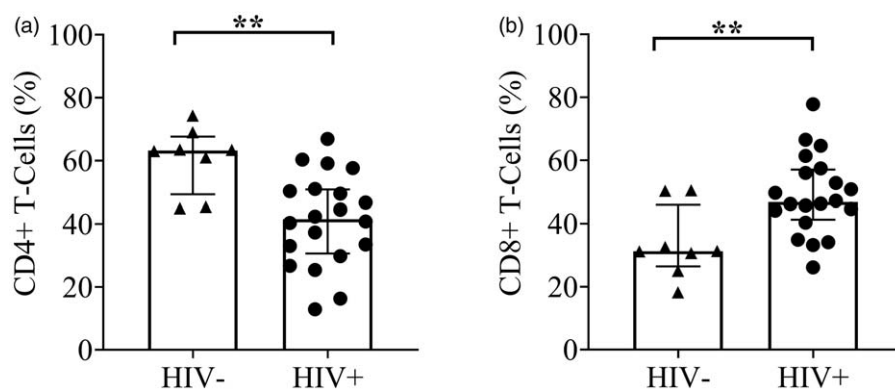


Fig. 1. CD4⁺ and CD8⁺ T cells as a percentage of live CD3⁺ T cells. Eight HIV-1-negative donors and 20 HIV-1-positive donors were stained with Live/Dead, CD3⁺, CD4⁺, CD8⁺ antibody stains and acquired. (a) CD4⁺ T cells; (b) CD8⁺ T cells. Triangles represent HIV-1-negative donors, circles represent HIV-1-positive donors. Mann-Whitney test performed to determine significance. A *P* value of less than 0.05 indicates significance, **P* < 0.05, ***P* < 0.001, ****P* < 0.0001. Error bars indicate median with interquartile range.

expression of IL-21R in HIV-1-positive and HIV-1-negative individuals. We assessed levels of IL-21R expression on CD4⁺ and CD8⁺ T cells from both groups and found that the levels of IL-21R were

significantly higher on the CD8⁺ T cells from HIV-1-positive donors relative to HIV-1-negative donors but no significant difference on CD4⁺ T cells (*P* = 0.0256) (Fig. 3a and b). This significant difference in expression

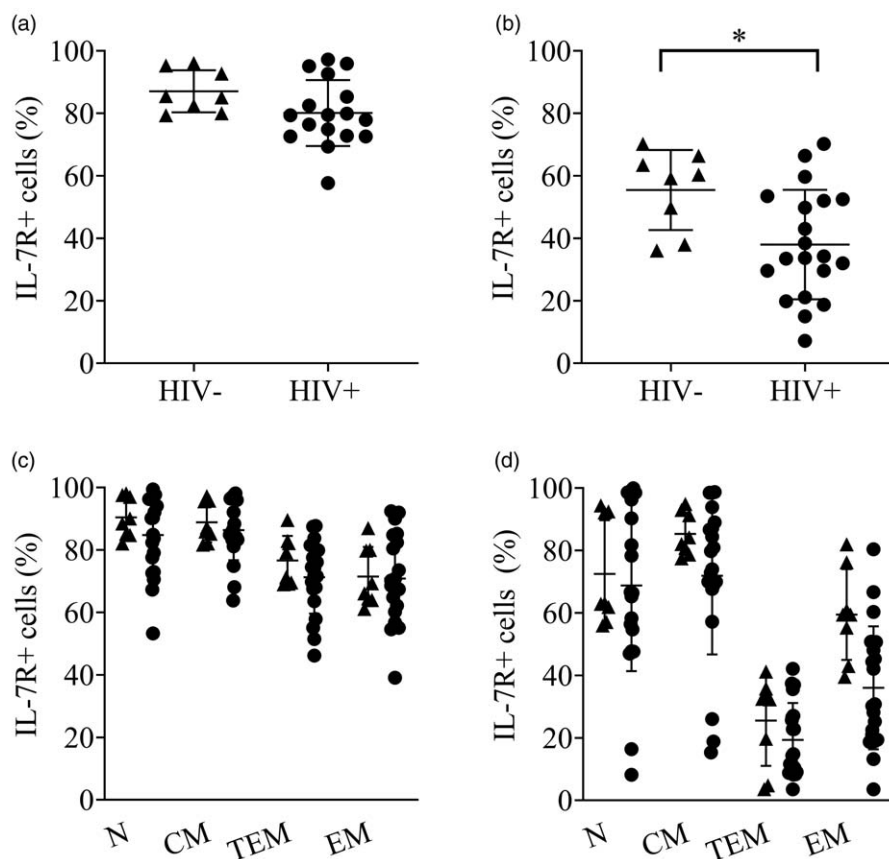


Fig. 2. IL-7R expression on total CD4⁺ and CD8⁺ T cells and T-cell subpopulations. Twenty HIV-1-positive and eight HIV-1-negative donor PBMCs were stained. IL-7R expression on (a) total CD4⁺ T cells, (b) total CD8⁺ T cells, (c) CD4⁺ and (d) CD8⁺ T-cell subpopulations. N, naive subset (CCR7⁺, CD45RO⁻); CM, Central Memory subset (CCR7⁺, CD45RO⁺); TEM, Terminal effector subset (CCR7⁻, CD45RO⁻); EM, Effector Memory subset (CCR7⁻, CD45RO⁺). Triangles represent HIV-1-negative donors, circles represent HIV-1-positive donors. Mann-Whitney test performed to determine significance. A *P* value of less than 0.05 indicates significance, **P* < 0.05, ***P* < 0.001, ****P* < 0.0001. Error bars indicate median with interquartile range.

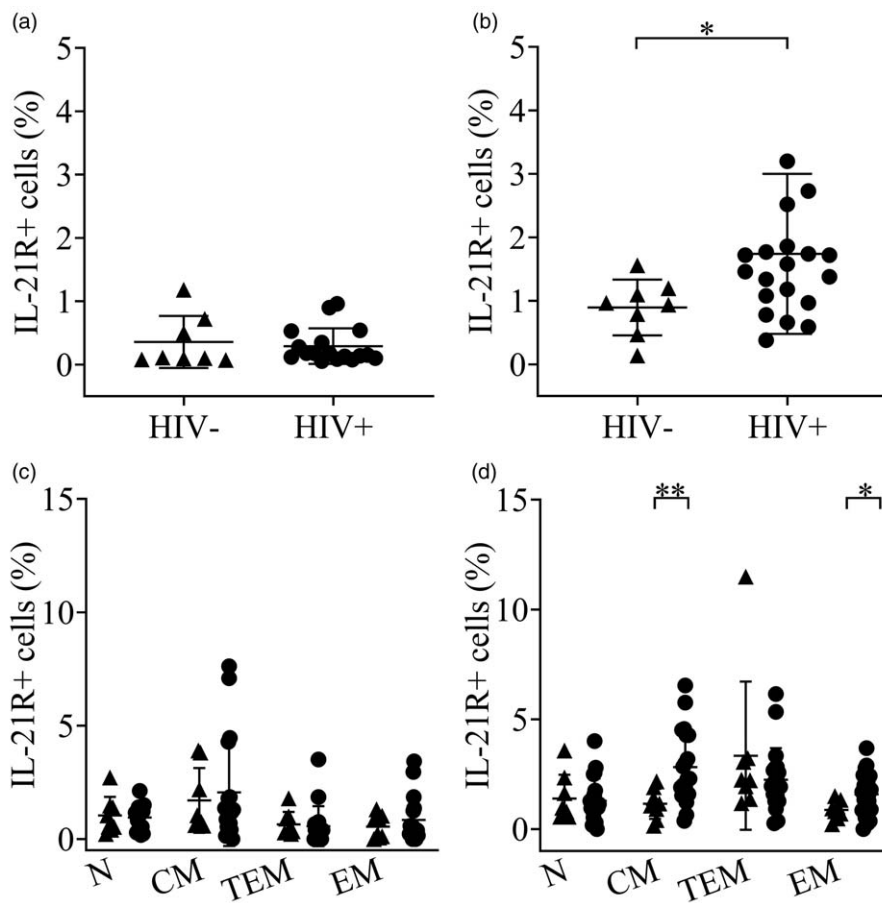


Fig. 3. IL-21R expression on total CD4⁺ and CD8⁺ T cells and T-cell subpopulations. Twenty HIV-1-positive and 8 HIV-1-negative donor PBMCs were stained. IL-21R expression on (a) total CD4⁺ T cells, (b) total CD8⁺ T cells, (c) CD4⁺ and (d) CD8⁺ T-cell subpopulations. N, naive subset (CCR7⁺, CD45RO⁻), CM, Central Memory subset (CCR7⁺, CD45RO⁺), TEM, Terminal effector subset (CCR7⁻, CD45RO⁻), EM, Effector Memory subset (CCR7⁻, CD45RO⁺). Triangles represent HIV-1-negative donors, circles represent HIV-1-positive donors. Man-Whitney tests were performed to determine significances. A *P* value of less than 0.05 indicates significance, **P* < 0.05, ***P* < 0.001, ****P* < 0.0001. Error bars indicate median with interquartile range.

was also evident when comparing CD8⁺ T-cell subpopulations, with central memory and effector memory sub-populations in HIV-1-positive donors showing greater levels of expression of IL-21R, whereas the naive and terminal effector subsets showed no difference (naive *P* = 0.9714, central memory *P* = 0.0055, effector memory *P* = 0.0487, terminal effector *P* = 0.4321) (Fig. 3d). Our analysis of IL-21R expression on all CD4⁺ T cells showed no significant difference in expression levels between HIV-1-negative and HIV-1-positive individuals (Fig. 3c).

IL-7R and IL-21R expression on cytomegalovirus-positive and HIV-1-positive specific CD8⁺ T cells

We next determined IL-7R and IL-21R expression on virus-specific CD8⁺ T cells. All 20 HIV-1-positive individuals were stimulated with CMV and HIV-1 peptide pools prior to flow cytometry analyses, followed by gating on the total antigen-specific CD8⁺ T cells. Stimulation of PBMCs with CMV peptides, HIV-1

peptides or PHA resulted in significantly greater proportion of cytokine-secreting CD8⁺ T cells compared with Mock (unstimulated) negative controls (HIV-1 *P* = 0.0001, CMV *P* = 0.0005, PHA *P* = < 0.0001) (Fig. 4a) as expected. There was no significant difference in IL-7R or IL-21R expression between HIV and CMV-specific CD8⁺ T cells (*P* = 0.0781, *P* = 0.0773, respectively; Fig. 4b and c).

Correlation between plasma viral load of HIV-1-positive volunteers and IL-21R expression

To determine the extent to which the level of IL-21R expression is linked to HIV-1 infection, we compared the visit plasma viral load data for the HIV-1-positive volunteers with the levels of IL-21R expression on CD8⁺ T cells. We first looked at IL-21R expression of the total CD8⁺ T-cell population of HIV-1-positive individuals. A Spearman's correlation test comparing IL-21R expression of the total CD8⁺ T-cell population with visit viral load showed no significant relationship

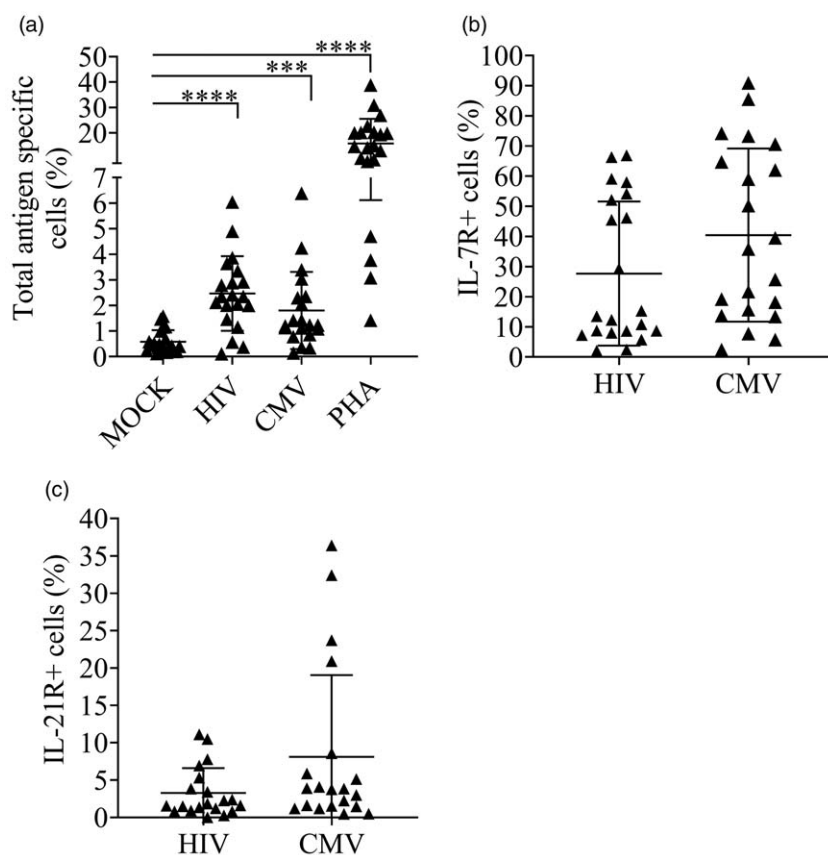


Fig. 4. Receptor expression levels on antigen (CMV or HIV-1)-specific CD8⁺ T cells. (a) 20 HIV-1-positive PBMCs were stimulated with total HIV-1 (Gag, Pol, Env and Nef) peptide pools or CMV peptide pp65, Mock or PHA for 6 h at 37 °C. Total antigen-specific CD8⁺ T cells were determined by Boolean gating of IFN- γ +, TNF- α + and double-positive gates. The percent of HIV-1 or CMV-specific CD8⁺ T cells expressing IL-7R+ (b) and IL-21R+ (c) were determined. Man-Whitney tests were performed to determine significances. A *P* value of less than 0.05 indicates significance, **P* < 0.05, ***P* < 0.001, ****P* < 0.0001. Error bars indicate median with interquartile range.

(Supplementary Fig. 2, <http://links.lww.com/QAD/C60>). However, when we focused exclusively on IL-21R expression on the HIV-1-specific CD8⁺ T-cell population, we found a significant positive correlation between receptor expression and plasma viral load at the time of PBMCs collection (*P* = 0.0152, *r* = 0.6667) (Fig. 5a).

HIV-1 disease progression and IL-21R expression

We measured IL-21R expression on CD8⁺ T cells from 11 treatment-naïve HIV-1-positive individuals whose disease progression has been well defined and characterized previously in a longitudinal study [37]. Set point viral load has been characterized as part of a separate investigation [34]. Three individuals, classified as low viral load controllers, had set point viral loads between 49 and 1279 copies/ml (Supplementary Table 1, <http://links.lww.com/QAD/C60>). Four of the 11 individuals showed poor disease control with set point viral loads between 72 990 and 503 486 copies/ml (chronic progressors). The remaining four individuals had ‘intermediate’ set point viral loads (1595– 28 929 copies/ml)

(intermediate controllers). We assessed IL-21R expression on CD8⁺ T cells from these 11 donors and found a trend for increasing receptor expression with disease progression (Fig. 5b).

Circulating IL-21 levels are associated with IL-21 receptor expression and HIV-1 disease progression

To determine if plasma IL-21 is associated with IL-21R expression, we quantified plasma IL-21 levels for all 11 Protocol C volunteers and compared it with IL-21R expression on total CD8⁺ T cells. There was no significant difference between the levels of IL-21 in plasma and IL-21R expression on the total CD8⁺ T-cell population (Supplementary Figure 2, <http://links.lww.com/QAD/C60>). However, there was a statistically significant inverse correlation between circulating IL-21 and IL-21R expression on HIV-specific CD8⁺ T cells (*P* = 0.0440, *r* = -0.6273) (Fig. 5c). We also compared disease status based on set point viral load and the levels of IL-21 in plasma for the same donors and found a trend of

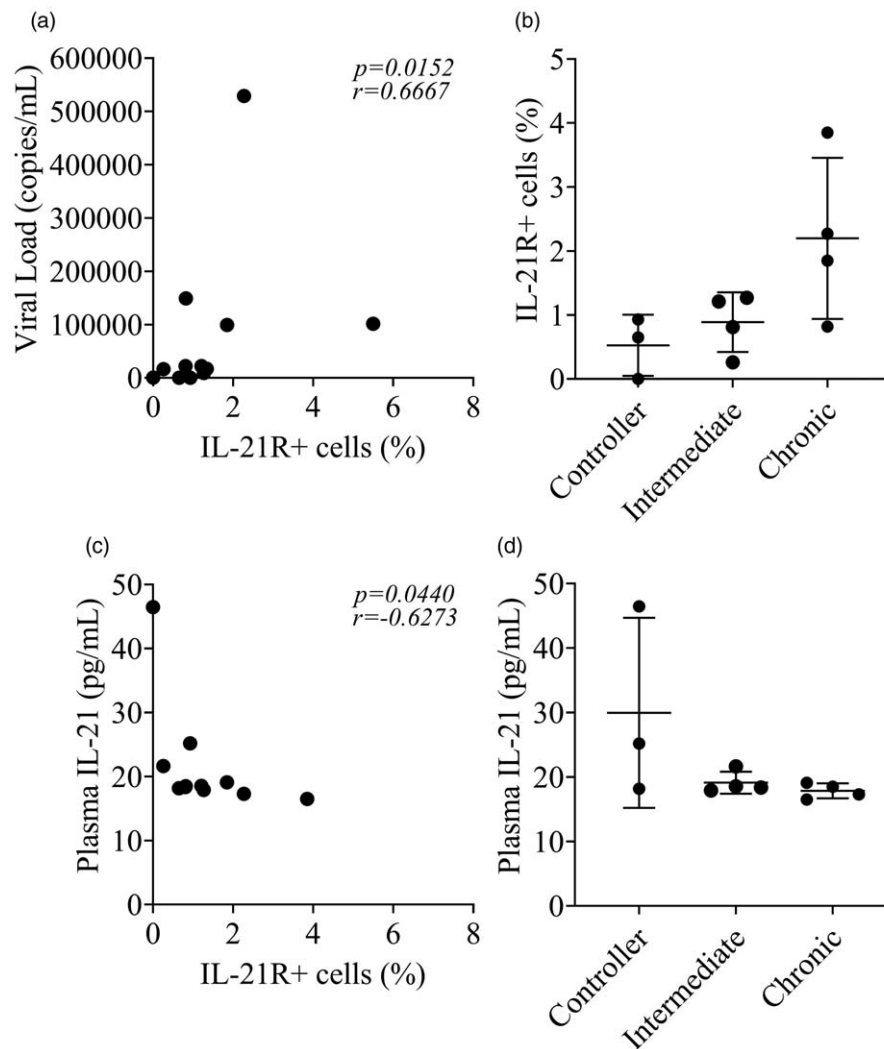


Fig. 5. Correlation of viral load, plasma IL-21 and IL-21R expression on HIV-specific CD8⁺ T cells (a and c) or against disease status based on set point viral load (b and d). PBMCs from HIV-1-positive volunteers ($n = 13$) were stimulated with total HIV-1 peptide pools (Gag, Pol, Env, Nef) or MOCK negative control for 6 h at 37 °C. IL-21R gated on total antigen-specific CD8⁺ T cells determined by Boolean gating of IFN- γ +, TNF- α + and double-positive gates. Plasma IL-21 measured in plasma samples from IAVI Protocol C donors ($n = 11$) in commercial ELISA IL-21 kit following manufacturers protocol. Spearman correlation test was performed to derived P value and r score. P value of less than 0.05 indicates significance.

decreasing plasma IL-21 with disease progression (Fig. 5d).

Discussion

Whilst experiments in mice have shown the importance of IL-21R in murine viral infections [29–32], the role of IL-21R in the pathogenesis of human viral infections like HIV-1 is less clear. Our results show a statistically significant difference in IL-21R expression on CD8⁺ T cells derived from HIV-1-positive volunteers compared with HIV-1-negative individuals. We describe phenotypically distinct

CD8⁺ T-cell subsets, which are IL-21R^{high} that correlate with HIV-1 disease progression and decreased levels of plasma IL-21. Our findings demonstrate divergent trends for IL-7R and IL-21R expressions in infected vs. uninfected individuals.

Our results are in agreement with the current literature showing downregulation of IL-7R expression on CD8⁺ T cells as a result of HIV-1 infection [25–27]. This downregulation has previously been shown to correlate with exhaustion; defined as loss of IFN- γ production and enhanced apoptosis following sustained and persistent exposure to antigen [38]. HIV-1 *Tat* protein has been associated with this IL-7R downregulation on HIV-1-

specific CD8⁺ T cells [39]. It is also well known that IL-7 acts directly to downregulate the IL-7 receptor on CD8⁺ T cells [40–42] with increasing plasma levels of IL-7 correlating with decreasing expression of IL-7R on CD8⁺ T cells [43,44]. Taken together these observations highlight the variable factors that can act to regulate IL-7R expression, with HIV-1 infection directly contributing to its dysregulation.

We demonstrate a significant difference in IL-21R expression on CD8⁺ T cells derived from HIV-1-positive individuals compared with HIV-1-negative individuals. In contrast to the pattern observed for IL-7R (where HIV-1-positive CD8⁺ < HIV-1-negative CD8⁺), we show that the frequency of IL-21R-expressing CD8⁺ T cells is significantly higher for HIV-1-positive donors than HIV-1-negative volunteers. Although both cytokines are related so much as they share a common gamma cytokine receptor chain (γ_c) [15,16], IL-21 is predominantly secreted by CD4⁺ T cells [15,45,46] whilst IL-7 is produced by stromal and epithelial cells [47,48]. Both cytokines have been shown to contribute to proliferation, differentiation, maintenance, survival and cytotoxicity of CD8⁺ T cells during HIV-1 infection [18,19,46,49]. Adoro *et al.* [50] demonstrated the importance of IL-21 in HIV-1 infection, showing that the treatment of CD4⁺ T cells with IL-21 resulted in reduced susceptibility to HIV-1 infection in humans. They concluded that this IL-21-mediated inhibition is linked to the upregulation of miR-29 in CD4⁺ T cells, which are highly conserved miRNAs implicated in the inhibition of HIV-1 replication.

A defining feature of HIV-1 infection is the rapid depletion of CD4⁺ T cells, which are a primary source of IL-21 [45,46,51]. Studies have also shown that IL-21 secretion correlates with CD4⁺ T-cell depletion during HIV-1 infection [52,53]. Iannello *et al.* [52] showed that elite HIV-1 controllers are able to maintain normal plasma levels of IL-21 and that highly active antiretroviral therapy resulted in partial recovery of plasma IL-21 levels in infected individuals. Unlike IL-21, the cellular sources of IL-7 are not rapidly depleted as a result of direct targeting by HIV-1 infection. Furthermore, circulating IL-7 and HIV-1 *Tat* protein have been shown to act to downregulate IL-7R expression on CD8⁺ T cells in HIV-1 infected individuals [39–42].

Our observation of a significant inverse correlation between circulating levels of plasma IL-21 and IL-21R expression on HIV-specific CD8⁺ T cells isolated from treatment-naïve volunteers supports the notion that significantly higher levels of IL-21R could be due in part to decreased frequencies of CD4⁺ T cells and consequently, levels of circulating IL-21. Previously published data by Chevalier *et al.* [54] demonstrated that HIV-1-specific effector CD8⁺ T cells have an enhanced ability to inhibit viral replication *in vitro* after exposure to IL-21. Combined with our data on viral load and disease

progression, this suggests that the effect of CD4⁺ and consequently IL-21 depletion may have a direct impact on the antiviral potency of circulating HIV-specific CD8⁺ T cells. The study by Iannello *et al.*, which showed an inverse correlation between viral load and plasma IL-21 underscores our data showing a trend of decreasing plasma IL-21 and disease progression.

We found no significant difference in IL-21R expression between HIV-1 and CMV-specific CD8⁺ T cells from HIV-1 and CMV-positive volunteers. This is an interesting observation considering that previously published data suggests that HIV-1-specific CD8⁺ T cells could be more sensitive to IL-21 than CMV-specific CD8⁺ T cells [49]. It is worth highlighting the differences in the sample type and size of the two studies and the differences in methodology. In addition, Yue *et al.* [55] use HIV-1 and CMV tetramers, which are known to capture very specific populations of T cells while being less sensitive to lower affinity cells. By using overlapping peptides covering the majority of HIV-1 and CMV CD8⁺ T-cell epitopes, our study potentially bypasses the need to consider MHC Class I restrictions in its characterization of antigen-specific populations.

Our study shows an association between the ability to control in-vivo viral replication (in the 11 Protocol C controllers and progressors) and IL-21R expression on HIV-1-specific CD8⁺ T cells. There is also a significant positive correlation between IL-21R expression on HIV-1-specific CD8⁺ T cells and the visit viral load. Although decreased plasma IL-21 appears to be associated with increased levels of IL-21R expression on HIV-1-specific CD8⁺ T cells, it is also possible that direct and continuous antigen stimulation seen in chronic viral infections could also play a role. Wu *et al.* [56] stimulated human PBMCs with PMA and ionomycin and showed a significant increase in IL-21R mRNA levels after 2 h of stimulation. Although Chevalier *et al.* [54] observed that incubation with optimal HIV-1 peptides resulted in increased IL-21R expression on HIV-1-specific CD8⁺ T cells from chronic progressors. These observations highlight the significance of further studies to assess the dynamics of cytokine–cytokine receptor interactions and what we can learn through longitudinal studies in the context of persistent viral infection.

Both vaccine and cure strategies based on T-cell-mediated immune responses against HIV-1 will require the effective expansion and survival of HIV-specific CD8⁺ T cells. In AIT for murine tumours, the expansion and total numbers of CD8⁺ T cells correlated with the degree of tumour regression observed [57]. This significant expansion was attributed to IL-21 in combination with IL-7 and IL-15 [58]. IL-21 has also been linked with CD8⁺ T-cell survival and decreased exhaustion as measured by a decrease in PD-1 expression and the formation of long-lived central memory T cells [59].

Exploiting T-cell-based therapies for HIV-1 treatment may require a thorough understanding of the role of IL-21, and the potential of the IL-21–IL-21R signalling in HIV-specific T-cell expansion and survival.

In conclusion, our data identifies CD8⁺ T-cell subsets with an IL-21R^{high} phenotype that correlates with viral load and lack of HIV-1 control. This observation may be explained by HIV-1 targeting of IL-21-secreting CD4⁺ T cells leading to their depletion. We suggest that circulating IL-21 may be capable of modulating the IL-21 receptor in the same manner as has been demonstrated for IL-7. The data also highlights a possible role for IL-21 and its receptor in optimal CD8⁺ T-cell-mediated control of viral infections [54]. Combined with our data on viral load and disease progression, this suggests that the effect of CD4⁺ and consequently IL-21 depletion may have a direct impact on the antiviral potency of circulating HIV-specific CD8⁺ T cells. Further experimentation will also be required to confirm whether IL-21 expression could be dysregulated by the continuous stimulation of CD8⁺ T cells by HIV-1 antigens, as has been shown for the IL-7R [39]. Although the sample size of this study is small, the relationships observed are statistically significant. We suggest that IL-21R could be a marker of disease progression for functional subsets of CD8⁺ T cells in HIV-1 infection. Determining the mechanism by which IL-21R expression is regulated on CD8⁺ T cells during HIV-1 infection may prove to be of great importance.

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Authorship: all authors have read and approved the final manuscript. J.D. performed the research and was responsible for conceptualization, sample application, methodology, data curation and analysis, original draft preparation, review and editing. J.M. was involved in sample application, methodology development, assay review, data curation and original draft review and editing. S.K.U. was involved in running flow cytometry assays, flow data curation and original draft review. P.H. was involved in Protocol C sample preparation and original draft review. L.B. was involved in flow cytometry antibody-stain panel design. D.K. and S.J. were involved in original draft review. J.G. was one of the lead investigators on IAVI protocol C study, and in the current study design, methodology review, original draft review and editing. The IAVI protocol C investigators were responsible for the initiation and successful completion of the Protocol C study.

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Conflicts of interest

There are no conflicts of interest.

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